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Highly pure preparations of recombinant gp120 were obtained from two different HIV-1 isolates. Conjugates of these HIV-1 gp120 proteins were prepared with tetanus toxoid (TT) and keyhole limpet hemocyanin (KHL) with the hypothesis that very immunogenic carrier proteins will induce better antibody responses to gp120. Conjugation procedures were selected that did not affect the ability of HIV-1 gp120 to bind to CD4. However, immunization studies in mice showed that anti-gp120 antibody levels were lower in animals immunized with conjugates compared with animals receiving HIV-1 gp120 alone. Moreover, repeated immunizations with either KHL-gp120 conjugates or gp120 alone failed to induce neutralizing antibodies to an HIV-1 clade B isolate. A combination of DNA vaccination and purified HIV-1 gp120 booster immunization was tested in baboons. The animals did not respond to the DNA vaccination, even in the presence of co-inoculated cytokines that are known to enhance the immune response. Only immunization with purified HIV-1 gp120 elicited a detectable, but short-lived, antibody response. Purified recombinant HIV-1 gp120 may not be the proper antigen for an effective vaccine against HIV-1.

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INTRODUCTION

It has been repeatedly demonstrated by a number of investigators that immunization of both humans and various animal models with antigenic products based on the envelope glycoprotein of the human immunodeficiency virus type I (HIV-1) results, at best, in the generation of low neutralizing anti-HIV-1 antibody titers. These results clearly suggest that present HIV-1 envelope vaccine approaches may not be effective. In addition, the transient nature of the antibody responses and the predominance of isolate-specific neutralizing activity have also been discouraging to efforts aimed at developing an effective HIV-1 vaccine (Cohen 1993). Similar results confounded early investigators utilizing polysaccharide (Ps) and carbohydrate antigens as vaccine candidates for bacterial and parasite pathogens. These studies demonstrated weak immunogenicity especially in infants who were most susceptible to infections caused by these pathogens. This promoted a number of conjugate vaccine studies in which the Ps antigen with low immunogenicity was covalently coupled to a highly immunogenic protein antigen (i.e., tetanus toxoid, the outer membrane protein complex of *Neisseria meningitides* (ONTC), diphtheria toxoid, among others) (reviewed in Vella and Ellis 1992). These early Ps conjugate-based vaccines were designed to activate a carrier-specific T lymphocyte response, which in turn drives B-lymphocytes toward the production of antibodies specific for the non-immunogenic target antigen or hapten; this is commonly referred to as the well characterized hapten-carrier phenomenon. The conjugate-based vaccines have met with mixed success in experimental animal models. In some instances, conjugated antigens have proven highly immunogenic in promoting the desired enhancement of anti-pathogen antibody responses, whereas, in other studies the addition of a carrier antigen significantly suppressed the desired antibody response (Herzenberg et al., 1980; Schutze et al., 1985). Over the past several years, however, conjugate vaccines have been more successful and a number of these vaccines have received United States Food and Drug Administration (FDA) approval for use in adults and infants (i.e., *Haemophilus influenzae* type B, group B *Streptococcus* type *111, Pneumococcus,* among others). Thus, it was the overall objective of this research project to assess the potential of an HIV-1 envelope glycoprotein (gp120) conjugate-based vaccine to induce high, long-lasting anti-HIV-1 envelope antibody titers with group specific neutralizing activity against primary and laboratory isolates of HIV-1.

RESULTS

Specific Aim #1: *Produce and characterize 100 mg of HIV-1 gp120 derived from the LAI and JR-FL strains for use in conjugation and immunization experiments.*

Development and culture of cell lines expressing recombinant HIV-1_{LAI} and HIV-1_{JR-FL} gp120s:

The nucleotide sequences encoding the laboratory-adapted T-cell tropic HIV-1_{LAI} gp120 protein or the primary macrophage tropic isolate HIV-1_{JR-FL} gp120 protein were inserted into the optimized mammalian expression vector PPI3-gpl20, where gpl20 expression is under the control of the cytomegalovirus MEI promoter and enhancer regions. The vector also encodes the gene for dihydrofolate reductase (dhfr), which imparts resistance to methotrexate (MTX). PP13 -gp120 was transfected into DXB-11 dhfr-CHO cells. Positive clones were selected for growth in nucleoside-free media, amplified by stepwise increases in the concentrations of MTX, and adapted for growth in reduced serum media. For large-scale production, fifty roller bottles were seeded with clones secreting gp120 from either virus strain and grown to confluence. Supernatant collections were performed twice weekly in ExCell 301 media (JRH Biosciences) containing 2% fetal bovine serum (FBS, Gibco-BRL). Greater than 50L of conditioned media were collected, sterile filtered, and stored frozen at -95 °C prior to purification. Each gp120 protein was expressed at 5-10 mg/L for a total of 250-500 mg prior to downstream processing.

Purification of recombinant HIV-1_{LAI} gp120 and HIV-1_{JR-FL} gp120:

The optimized process was performed entirely at neutral pH in the absence of chaotropic or denaturing agents and was proven equally effective in purifying recombinant gp120 from either the LAI or JR-FL strain of HIV-1. Briefly, clarified roller bottle supernatant was passed over a *Galanthus nivalis* (GNA) column. The GNA lectin recognizes glycans containing terminal mannose residues, which are found extensively on native gp120 (Gilljam, 1993). After extensive column washes, glycoproteins were competitively eluted with 0.5M methyl-α-D-mannopyranoside in PBS. The GNA eluate was dialyzed against 20mM imidazole buffer at pH 7.1, loaded onto a Q High Performance anion exchange column (Pharmacia) equilibrated in the same buffer, and eluted with step-wise increases in sodium chloride concentration. The post-Q material was concentrated and loaded onto a Superdex 200 gel filtration column equilibrated in PBS. The entire purification process was carried out at 4 °C.

Purified gp120 was reduced with dithiothreitol (DTT), loaded onto a pre-cast 4-15% Ready Gel (Bio-Rad Laboratories), run on SDS-PAGE, and visualized with coomassie blue (Figure 1). The purified gp120 proteins ran as a single diffuse band with apparent molecular

weights near 120kd, as expected for proteins bearing native glycosylation patterns. In comparison HIV-1_{LAI} gp120, HIV-1_{JR-FL} gpl20 contains 13 fewer amino acids and one less site of potential N-linked glycosylation. As expected from these differences in primary sequence, recombinant HIV-1_{JR-FL} gpl20 ran with a measurably increased mobility on SDS-PAGE. The purity of each preparation was estimated as >95% using ImageQuant software (Molecular Dynamics). In total, the gel results suggested that the production process yielded gp120 preparations that were highly purified, free of degradation products, and possessed of native glycosylation patterns.

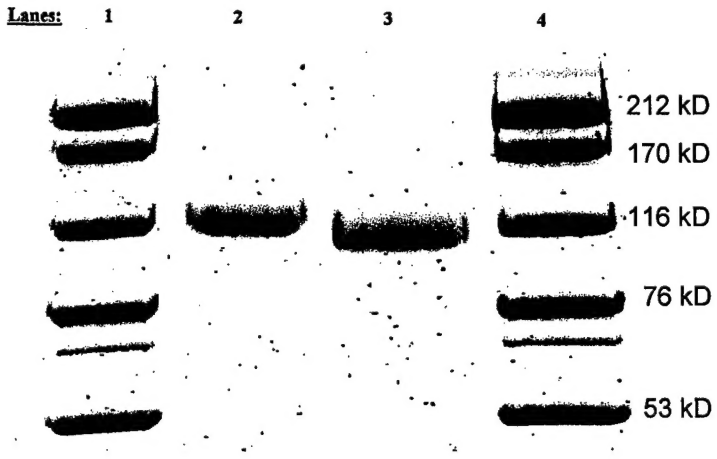

Figure 1. SDS-PAGE analysis of purified, recombinant HIV-1_{LAI} gp120 (lane 2) and HIV-1_{JR-FL} (lane 3). A total of 10μg of each protein were loaded on a 4-15% Ready-Gel and visualized with Coomassie Blue. Lanes 1 and 4 are Pharmacia high molecular weight markers.

Analysis of gp120 binding to CD4-based proteins:

The purified recombinant gp120 proteins from HIV-1_{LAI} and HIV-1_{JR-FL} were tested for their abilities to bind soluble CD4 (sCD4) and CD4-IgG2 in an ELISA format. CD4-IgG2 is a novel heterotetramer comprising two chains of a CD4-human IgG2 heavy chain fusion protein and two chains of a CD4-human K fusion protein; its expression, characterization, and anti-viral properties have been recently described (Allaway et al., 1995; Trkola et al., 1995). Briefly, gp120 was captured onto an ELISA plate (Dynatech Laboratories, Inc.) at 5 ng/well using a polyclonal antibody to the conserved C terminus of gp120 (International Enzymes, Inc.). The plate was blocked by incubating with 5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 2 hr. Purified sCD4 or CD4-IgG2 was then added in a range of concentrations

(e. g., 0.1-100 nM) to the immobilized gp120 and incubated for 1 hr at 37 °C. Following wash steps, the amount of bound sCD4 was determined by incubation with the mouse anti-CD4 monoclonal antibody OKT4 (Ortho Diagnostic Systems, Inc.), followed by incubation with horseradish peroxidase (HRP)-labeled goat anti-mouse antibody (Kirkegaard & Perry Laboratories, Inc.) and then with o-phenylenediamine (OPD) substrate. Bound CD4-IgG2 was detected by incubation with HRP-labeled affinity purified goat antibody specific for human Ig Fc, followed by OPD substrate. Absorbance readings were measured at 492 nm using an automated ELISA plate reader. All wash and incubation steps employed PTB buffer (0. 5% Tween 20, 1 % FBS, and 0.1 % BSA in PBS).

The binding data are summarized in Table I and are expressed as EC_{50} values, the concentration of CD4-based protein giving half-maximal binding. The EC_{50} value is a reasonable approximation of the thermodynamic dissociation constant (Orloff et al., 1993; Moore et al., 1991). The recombinant gp120 molecules from both the laboratory-adapted strain and the primary isolate bound to CD4-based proteins with nanomolar affinity. These results compare favorably with binding data obtained using gpl20 extracted from virions (Moore et al., 1990) and suggest that the purified recombinant proteins are conformationally intact.

Table I. Comparison of HIV-1LAI gp120 and HIV-1JR-FL gp120 binding to CD4-based proteins in an ELISA format

	EC_{50} (nM)	
CD4-based protein	**HIV-1_{LAI}**	**HIV-1_{JR-FL}**
sCD4	1.93	2.22
CD4-IgG2	1.25	0.64

Analysis of recombinant HIV-1_{LAI} gp120 and HIV-1_{JR-FL} gp120 binding to cell surface CD4:

To characterize the purified gp120 molecules further, their binding to cell surface CD4 was analyzed by flow cytometry. Each gp120 was incubated at 5 μg/ml for 2 hours at 37 °C in FACS buffer (PBS + 2% BSA + 0.1% sodium azide) with a CHO cell line engineered at Progenics to express high levels of full length human CD4. The cells were then washed and incubated with a sheep antibody to the conserved C-terminus of gp120. After another wash, the cells were incubated with FITC-conjugated rabbit anti-sheep Ig, washed and fluorescence determined using a Becton Dickinson FacScan. Only CHO cells expressing human CD4 stained positive for FITC, indicating proper binding of gp120 to CD4 (data not shown).

Analytical gel filtration analysis of purified HIV-1LAI gp120 and HIV-1JR-FL gpl20 proteins:

The aggregation state of the purified gp120 proteins was determined by analytical gel filtration on a TSK G3000SWXL column (TosoHaas) run in 100 mM sodium phosphate, 100 mM sodium sulfate buffer, pH 6.7. Both HIV-1_{LAI} gp120 and HIV-1_{JR-FL} gp120 proteins were >95% monomeric when purified using the optimized three-column method. Similar results were obtained for HIV-1_{LAI} gp120 purified using the two-column method (data not shown). The remaining material consisted primarily of high molecular weight species that have been tentatively identified as disulfide-linked gp120 aggregates that bind sCD4 poorly in ELISA.

Analytical gel filtration analysis of gp120-CD4 binding interaction:

The ELISA described above was useful in determining the strength of the gp120-CD4 interaction. However, in order to assess whether the majority of gp120 molecules present in our preparations were capable of binding to CD4, an analytical gel filtration binding assay was developed. In this assay, HIV-1_{LAI} gp120 was incubated with a slight molar excess of sCD4 for 1 hr at 4°C in PBS and then injected onto a TSK G3000SWXL column (TosoHaas) equilibrated in PBS. As indicated in Figure 2, the addition of sCD4 caused the gp120 peak at 6.2 min (Figure 2, middle profile) to disappear and a new peak to appear at 6.0 min (Figure 2, lower profile). The new peak is presumably the gp120-sCD4 molecular complex. In the profile for the mixture (Figure 2, lower profile), the absence of split peaks or even a defined shoulder at 6.2 min suggests that the gp120 preparation is essentially fully reactive with sCD4. The high specific activity of the preparation was attributed to the fidelity of the mammalian cell expression system and the mild conditions employed during purification.

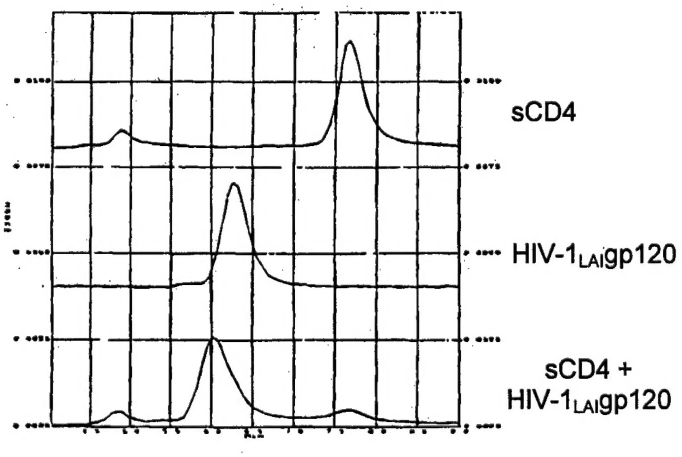

Figure 2. Gel filtration analysis of gp120-sCD4 binding.

Immunogenicity of unconjugated HIV-1_{LAI} and HIV-1_{JR-FL} gp120's in two adjuvants:

The HIV gp120 preparations were initially tested for immunogenicity in the native form, prior to conjugation. Guinea pigs (3 animals per group) were inoculated 5 times with 100μg of either gp120 strain in alum or Ribi Detox adjuvants. The immunization schedule included subcutaneous and intraperitoneal administrations. Bleeds were performed prior to and at intervals after the first injection. The humoral response was determined by ELISA. Briefly, gp120 of the same strain used for immunization was immobilized on an ELISA plate. Following blocking and washing steps, dilutions of serum from 1:8,000 to 1:512,000 in PBS/Tween were added. The level of bound antibody remaining after washing was determined using peroxidase-labeled goat anti-guinea pig Ig. The greatest serum dilution giving a signal twice the value obtained with pre-immune serum was defined as the titer. As shown in Figure 3, the reciprocal serum titers increased following sequential immunizations to a maximum of 250,000 to 500,000. Similar results were obtained using LAI and JR-FL gp120's. Moreover, while early titers were higher with the Ribi adjuvant, the maximum titers achieved using both adjuvants were similar.

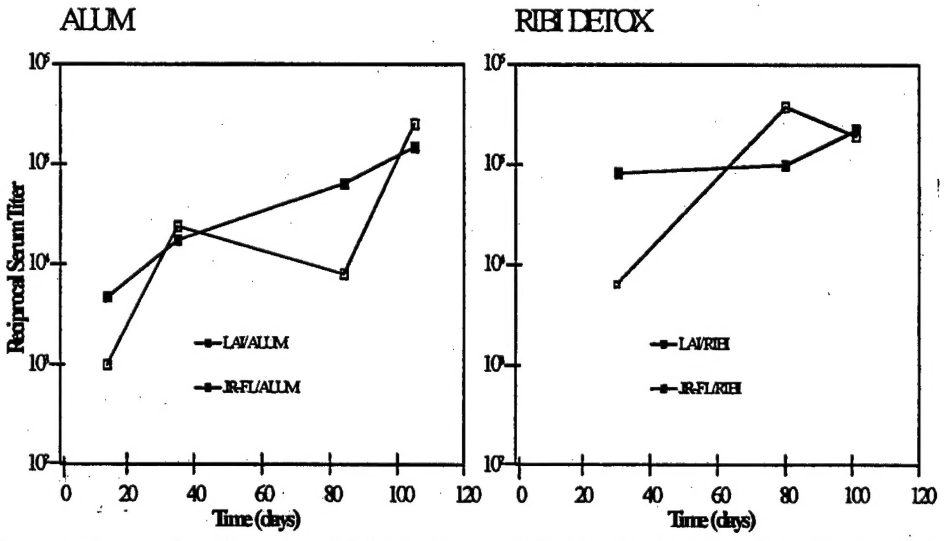

Figure 3. Comparison of anti-gp120 ELISA titers exhibited in Guinea pigs immunized with LAI and JR-FL gp120's.

Next we examined the sera from these immunized guinea pigs to determine the level of antibodies to the CD4-binding site of gp120, capable of blocking the gp120-CD4 interaction. Such antibodies would be expected to neutralize a broader range of HIV-1 strains than antibodies to other epitopes such as the V3 loop. Briefly, immune sera from the animals were dialyzed and the immunoglobulin fraction prepared by Protein A affinity chromatography.

ELISA plates were coated with sCD4, then incubated with biotinylated gp120 which had been pre-incubated (2 hours at room temperature) with dilutions of the immunoglobulin preparations. The amount of gp120 bound was determined using peroxidase-conjugated avidin and compared with the maximum bound in the absence of antibodies. The antibody dilution that inhibited binding by 50% was defined as the gp120-CD4 blocking titer. The results are shown in Table II, converted to the equivalent serum titer.

Table II. gp120-CD4 blocking titers generated in guinea pigs immunized with HIV-1_{LAI} gp120 and HIV-1_{JR-FL} gp120 proteins in two adjuvants.

Immunizing gp120	Adjuvant	Blocking titer against LAI gp120	Blocking titer against JR-FL gp120
LAI	Alum	381	345
LAI	Ribi Detox	2233	725
JR-FL	Alum	73	211
JR-FL	Ribi Detox	52	427

All sera contained antibodies capable of blocking CD4 binding, in each case being more potent in the assay using homologous gp120. Higher blocking titers were obtained in most cases using Ribi Detox as an adjuvant compared with alum, presumably because the latter denatures the immunogen, thereby inhibiting the generation of antibodies to conformational epitopes such as the CD4 binding site on gp120.

Specific Aim #2: *Conjugate tetanus toxoid and diphtheria toxoid carriers to the gp120 molecules using a variety of established chemistries. Evaluate both the integrity of gp120 after conjugation and the immunogenicity of the HIV-1 subunit conjugates in mice.*

The hypothesis was that the incorporation of highly immunogenic antigen carrier into HIV-1 gp120, a heavily glycosylated envelope protein, will result in a more active immunologic recruitment of helper T lymphocytes and will significantly augment the anti HIV immune response. In order to test such hypothesis, tetanus toxoid (TT) was conjugated to purified gp120 using glutaraldehyde and mice were immunized with this conjugate in alum adjuvant.

Preparation and Immunogenicity of HIV-1_{LAI} gp120 and HIV-1_{JR-FL} gp120 conjugates to tetanus toxoid:

Purified gp120 preparations were conjugated to tetanus toxoid (TT) using glutaraldehyde. The fixative glutaraldehyde is a non-specific, mildly denaturing agent that has proven as an efficient reagent for protein to protein coupling (Harlow and Lane, 1988). Briefly, TT and HIV-1 gp120 were mixed at a 2.5:1 ratio in a solution of glutaraldehyde in sodium phosphate, pH 6.0, for 2 hours at room temperature. Excess glutaraldehyde was removed by dialysis against borate buffered saline. The binding of the conjugates to CD4, assessed as discussed above, showed that the conjugates retained their binding to CD4 (data not shown).

The gp120 or gp120-TT conjugates were precipitated in aluminum hydroxide and used to vaccinate groups of BALB/c and C57BL mice. Three immunizations of 5 μg HIV-1_{LAI} or HIV-1_{JR-FL} gp120 each were given intramuscularly on a monthly basis and the antibody titers were determined by ELISA essentially as described above.

Table III. Antibody responses in BALB/c and C57BL mice immunized with unconjugated gp120 or gp120 conjugated to tetanus toxoid (gp120-TT) administered in alum.

Mouse Inbred Strain	No. of Mice	Immunogen	No. of Immunizations	End-Point Antibody Titers
C57BL/6	5	gp120/Alum[a]	3 [b]	5,870[c] (2,000-11,600)[d]
	3	gp120-TT/Alum	3	1,433 (750-2,150)
BALB/C	5	gp120/Alum	3	6,160 (3,200-10,400)
	4	gp120-TT/Alum	3	2,432 (163-6,800)

[a] 5.0 μg of gpl20 were given per injection.
[b] Immunizations were given monthly.
[c] Mean antibody titers.
[d] Range of antibody titers

The results given in Table III suggested that conjugation of HIV-1_{LAI} gp120 to TT elicited lower anti-gp120 antibody titers as compared to those induced by unconjugated gp120. The reason for this reduction in immunogenicity was unclear, since the conjugates retained their CD4-binding capacity. The most likely explanation was that TT originated the phenomenon of

carrier protein-induced immunosuppression. Similar results were obtained with HIV-1_{JR-FL} and are not shown.

Because of the relatively low anti-gp120 antibody titers in mice vaccinated with gp120-TT described above and the possibility of immunosuppression induced by pre-existing anti-TT antibodies in humans, we selected to conjugate gp120 to KLH for immunogenicity studies.

Use of Keyhole Limpet Hemocyanin (KLH) as a protein carrier to improve the immunogfenicity of gp120:

As discussed above, the gp120-TT conjugates exhibited poor immunogenicity when injected into mice and may be subject to carrier-induced immunosuppression in TT-exposed humans. Therefore, an alternative approach was used, whereby the gp120s were conjugated with KLH. As a carrier for conjugate vaccines, this protein has the advantage over tetanus toxoid in that individuals are unlikely to have a pre-existing immune response to the carrier alone, which could limit the efficacy of conjugate vaccination. Several studies have found that KLH can enhance the antibody response to antigens, often more effectively than other carrier proteins, possibly because its size and antigenic complexity aid antigen processing and recruitment of T cell help (Helling et al., 1994; Longenecker et al., 1994). Clinical studies using various conjugates incorporating KLH as cancer vaccine candidates have demonstrated improved humoral immune responses and clinical benefits following treatment with these conjugates (Longenecker et al., 1994; Hefling et al., 1995).

Our studies employed a KLH preparation (PerImmune, Inc.) which has been administered to human subjects under IND's from multiple companies, including Progenics. In SDS-PAGE, the protein migrates as a major band at approximately 275kd. In nondenaturing assays such as sucrose gradient centrifugation or gel filtration, the protein migrates as an $8.6x10^6$ Dalton noncovalent multimer.

Preparation of gp120-KLH conjugates:

We examined several conjugation chemistries which target various functional groups of gp120, including carbohydrate moieties, primary amines, and carboxyl groups. To minimize modification of the gp120 protein, two-step procedures were followed whenever possible whereby KLH was the protein modified more extensively. The conjugates were analyzed for CD4-binding activity in a sandwich ELISA using anti-KLH antibodies to capture the conjugate. This ELISA is specific for gp120-KLH conjugates in that both KLH and gp120 epitopes must be present on the same molecule for a positive reaction to occur. In addition, the conjugates were

analyzed by SDS-PAGE to determine the extent of cross-linking. HIV-1_{LAI} gp120 was used in all of the conjugation studies described below.

1- SDBP: N-hydroxysuccinimidyl-2-3-dibromopropionate (SDBP) is a heterobifunctional crosslinking agent containing two amine-reactive moieties that vary in temperature sensitivity. That is, the reactivity of the alkylhalide group is nearly negligible at 4 °C but greatly improved at 25 °C, while the succinimide moiety is reactive throughout this temperature range. SDBP yields conjugates linked by a stable aziridine group and a 2-carbon spacer element. SDBP was used to crosslink KLH and gp120 in the following two-step procedure. In the first step, carried out at 4 °C, 2-4 mg of SDBP were dissolved in 200 µl of DMSO, buffered with 40 µl of PBS, and then added to 2mg KLH in 400 µl PBS. After a 2-hr reaction favoring the N-hydroxysuccinimidyl moiety of the crosslinker, KLH was dialyzed against PBS. In the second step, an equal mass of gp120 in PBS was added, and the mixture was warmed to ambient temperature to promote the reaction between alkylbromide-activated KLH and gp120. Following a 16-hour incubation, the conjugate was further dialyzed against PB S.

2- Glutaraldehyde: In addition to the expected Schiff base formation, glutaraldehyde-mediated crosslinking is thought to proceed by mechanisms involving unsaturated glutaraldehyde polymers (Wong, 1991). These Michael-type addition products are stable in the absence of reducing agents such as sodium borohydride. Both one- and two-step glutaraldehyde coupling procedures were examined (Hermanson, 1996). In the more successful two-step procedure, KLH was reacted for 1 hr with 0.075% glutaraldehyde in 50 mM phosphate buffer at pH 7.5 and then dialyzed against PBS at 4 °C. At this time, gp120 in PBS was added at an equal mass ratio and reacted for 2-hr at 4 °C. Tris was then added to 20mM to block any remaining active sites and the reaction was continued for an additional hour at 4°C, after which the conjugate was dialyzed against PBS.

3- Other procedures: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) conjugation reactions were carried out in one- and two-step procedures in the presence and absence of sulfo-N-hydroxysuccinimide. The protocols targeted both primary amino and carboxyl groups on gp120. Since gp120 glycans are reportedly dispensable for CD4-binding (Fenouiflet et al. 1989, 1996), conjugates were also initially prepared using reductive amination under a variety of test conditions. In the basic procedure, vicinal diols on gp120 glycans were oxidized to aldehyde groups using sodium m-periodate at neutral to slightly acidic pH values. Oxidized gp120 was combined with KLH under neutral to slightly basic conditions, and the resulting Schiff bases were reduced with either sodium cyanoborohydride or amine boranes, the latter being equally effective but less toxic reducing agents (Cabacungan et al, 1982).

Analysis of gp120-KLH conjugates for CD4 binding activity:

Conjugates were tested in a sandwich ELISA specific for gp120-KLH conjugates possessing a functional CD4-binding site. In the assay, Probind 96-well microtiter plates (Falcon) were coated with polyclonal rabbit anti-KLH antibodies (prepared by the Progenics Oncology program) at 100 ng/well. The plates were washed with PBS/0.02% Tween 20 (PBST) and blocked with PBS containing 5% BSA. To complete the assay, the following reagents were added with intermediate PBST washes: (1) gp120-KLH conjugate, typically at 50ng/well, (2) 2-150 nM sCD4, (3) the murine anti-CD4 monoclonal antibody OKT4 (Ortho Diagnostics), (4) HRP-conjugated goat anti-mouse IgG (Kirkegaard & Perry), and (5) OPD substrate. Signals were read at 492 nm on an ELISA plate reader, and the data were fit to a Langmuir isotherm using SlideWrite Plus. Background readings were routinely obtained in wells containing gp120, KLH, or mixtures of unconjugated gp120 and KLH. Conjugate binding properties were critically affected by crosslinking conditions, although appreciable CD4-binding activities were observed for conjugates prepared with SDBP and with glutaraldehyde. The estimated equilibrium constants ranged from 24 nM to 66 nM and were approximately 10-fold higher than those observed for monomeric gp120 in similar assay systems. As neither conjugation procedure is site-directed, the CD4-binding site was expected to be variably accessible in the conjugated molecules, reducing the average affinity of the interaction. The highest CD4 binding activity was observed for conjugates prepared using 3 mg/ml SDBP; conjugates prepared using 6 mg/ml SDBP were inactive. Similarly, the two-step glutaraldehyde procedure was more effective at 0.075% glutaraldehyde than at higher crosslinker concentrations. A one-step glutaraldehyde cross-linking procedure was ineffective at 0.25% glutaraldehyde. One possibility is that at higher concentrations of crosslinker, the gp120 molecule becomes conjugated through multiple covalent bonds that distort the CD4 binding site.

Additional chemistries failed to yield conjugates that were reactive in the above ELISA. Changes in periodate concentration, pH conditions, and reducing agent failed to yield CD4-reactive conjugates. Similarly, no significant CD4 binding activity was observed in gp120 self-conjugates prepared as above in the absence of KLH. The gp120 self-conjugates were analyzed in the EC_{50} assay described above which employs a polyclonal anti-gp120 coating antibody. This activity loss could reflect intramolecular linkages between oxidized glycans and critical lysine residues on gp120 or oxidation of periodate-sensitive amino acids such as methionine (Geoghegan and Stroh, 1992). In addition, no significant CD4-binding was observed for gp120-KLH conjugates prepared using EDC. Although the conjugates lacked

detectable CD4-binding activity, SDS-PAGE analysis of the conjugates indicated partial to complete coupling of gp120 to KLH (data not shown), suggesting that the procedures altered or masked the CD4-binding site on gp120.

SDS-PAGE analysis of gp120-KLH conjugates:

Conjugates possessing measurable CD4-binding activity were analyzed by SDS-PAGE to assess the extent of crosslinking. As expected, the crosslinked species were too large to enter the gel, but the degree of crosslinking could be estimated from the intensity of the unconjugated gp120 and KLH bands. KLH was essentially completely crosslinked into high molecular weight species that were nearly completely excluded from the gel. Under the nondenaturing conditions of the conjugation procedures, KLH existed as a large multimeric protein, whose members would be expected to undergo intermolecular crosslinking. By comparing the intensities of the monomeric gp120 bands in the standard and conjugate lanes, we estimated that 50-90% of the gp120 was crosslinked in the conjugates prepared using SDBP and glutaraldehyde. Increasing the glutaraldehyde concentration from 0.075% to 0.25% produced an elevation in the degree of crosslinking, but only at the expense of reduced CD4-binding activity.

Specific Aim #3: *Identify the optimal HIV-1 subunit conjugate vaccine and extensively characterize the immune response (and in vitro anti-viral effects) in mice and baboons.*

Immunogenicity Studies In Mice:

To evaluate the immunogenicity of the KHL-HIV-1_{LAI} and -HIV-1_{JR-FL} gp120 glycoconjugate preparations, we immunized groups of BALB/c mice and determined anti-gp20 endpoint titers by methods as previously described (Wolf et al, 1992). Mice received multiple injections at monthly intervals of the gp120-KHL preparations in alum (5μg of gp120). The anti-gp120 titers were compared to the ones obtained in of mice immunized with the 5 μg of gp120 preparation in alum or saline. The immunogenicity of the gp120 preparations was increased by employing alum as an adjuvant (Table IV). IgG anti-gp120 titers were observed following the third (HIV-1_{LAI} gp120) and second (HIV-1_{JR-FL} gp120) immunization with the two different preparations. The anti-gp120 responses peaked following the fifth injection, and the anti-HIV-1_{LAI} gp120 titers were boosted by additional vaccinations. The anti-gp120 response in the HIV-

$1_{JR\text{-}FL}$ gp120 group remained stable following additional injections. These responses were compared to immunization with the glycoconjugates. Based on anti-gp120 titers, conjugation to KLH did not appear to enhance the immunogenicity of gp120 preparations. In fact, for both gp120 preparations, conjugation to KLH appeared to reduce immunogenicity based on anti-gp120 titers.

In summary, KLH-gp120 conjugates were prepared by methods that still allowed for CD4-binding; however, immunogenicity studies in mice showed that these conjugates were less immunogenic than unconjugated gp120 preparation.

Table IV. Anti-HIV-1 JR-FL and LAI gp120 endpoint titers in sera from immunized mice.[a]

Group[b]	Injection Number	Anti-gp120 titer[c] (LAI)	Anti-gp120 titer (JR-FL)
gp120 soluble	1	<50	<50
	2	<50	<50
	3	<50	<50
	4	<50	<50
	5	230(50-800)	2,680(100-6,400)
	6	ND	ND
	7	ND	ND
	8	710(50-3,200)	3,220(100-6,400)
gp120 alum	1	<50	<50
	2	<50	4,240(400-6,400)
	3	1,010(50-3,200)	40,960(25,600-102,400)
	4	5,960(200-12,800)	37,120(6,400-102,400)
	5	8,960(3,200-12,800)	71,680(25,600-102,400)
	6	ND	ND
	7	ND	ND
	8	32,320(1,600-102,400)	30,720(25,600-102,400)
KLH/gp120 alum	1	<50	<50
	2	<50	1,320(200-3,200)
	3	710(50-3,200)	6,880(400-25,600)
	4	3,530(50-12,800)	32,320(1,600-102,400)
	5	2,090(50-3,200)	37,120(6,400-102,400)
	6	ND	ND
	7	ND	ND
	8	4,200(400-6,400)	21,760(6,400-25,600)

[a] Titers were determined indirectly by solid phase ELISA.
[b] Groups consisted of 5 female Balb/c mice, immunized and bled monthly.
[c] The values represent the reciprocal of the dilution of sera that was greater than 3 times the value of the individual pre-sera less background. The mean of each group is shown with the range of the individuals in parentheses.

Neutralization of different HIV-1 isolates:

Prior to examining the ability of the immune mouse sera to inhibit the various primary isolate clades, it was necessary to determine the kinetics of HIV-1 replication in human PBMC. HIV-1 primary isolates were obtained from Quality Biologicals Inc., Gaithersburg, MD, the Aaron Diamond AIDS Research Center, New York, NY, and the AIDS Research and Reference Reagent Program, Rockville, MD. The following HIV-1 isolates were used in neutralization assays: UG029 (Uganda) subtype A, BZ167 (Brazil) subtype B, IN905 (India) subtype C, UG001 (Uganda) subtype D, and KH005 (Thailand) subtype E. Each of the isolates induced syncitium in PBMC and the stock titers ranged from 1×10^2 to 1×10^5 $TCID_{50}$/ml. Stocks were expanded in fresh human PBMC blasts by weekly co-culture. Inocula of 1, 10, and 100 $TCID_{50}$/ml were tested and samples were taken at days 4, 7, and 10, and assayed by p24 antigen capture. Based on HIV-1 p24 levels, at 100 $TCID_{50}$/ml inoculum, viral titers were maximum and constant from days 4, 7, and 10. At 10 $TCID_{50}$/ml the viral titers steadily increased from day 4 to day 10. At 1 $TCID_{50}$/ml the titers were not sufficient even at day 10. For evaluating the inhibitory activity of the anti-CD4 antibody, a 100 $TCID_{50}$/ml inoculum was selected for further studies. We performed similar studies with the other HIV-1 isolates that were utilized (data not shown) and also found 100 $TCID_{50}$/ml to be optimal.

Fresh human PBMC blasts were used as targets for HIV-1 primary isolate infections. Positive control samples were viral inocula pre-treated with 100 μg/ml P1. P1 is a murine IgG1/k antibody molecule that binds to human CD4 with an specificity similar to Leu/3a (Attanasio et al, 1993). Before testing for inhibition, it was necessary to test whether the immune mouse sera, pre-immune mouse sera and or P1 had any toxic effect on the PBMC blasts. In order to evaluate toxicity we used the MTT cell viability assay. No significant decrease in viability of the PBMC was observed in the presence of mouse sera at a 1:20 dilution or P1 at 100 μg/ml (data not shown). The results indicated that mouse sera and P1 were not toxic to the PBMC blasts that were going to serve as targets for subsequent infectivity assays.

Viral inocula (100 $TCID_{50}$) from representative clade A through E isolates were incubated with or without a 1:20 dilution of mouse pre-immune or vaccine sera for 1 hour prior to infection. Fresh 3-day blasted human PBMC, 2×10^6 per experiment, were infected with the pre-treated viral inocula. The infections were shaken every 15 minutes during the 2 hour adsorption period after which the PBMC were pelleted and unadsorbed virus was washed away with saline. Finally, the infections were resuspended in 2 ml of RPMI/IL-2 medium and added to individual wells of 24-well tissue culture plates. Infections were fed on days 4, 7, and 10 by

removing 1 ml of culture and replacing with 1ml fresh medium. The level of infectivity was determined based on OD values from the HIV-1 p24 ELISA (Warren et al, 1992). The data (Table) Indicated that the anti-JRFL vaccine sera from the alum precipitated gp120 and alum precipitated gp120 conjugates inhibited the primary clade B isolate by 33 and 15 % respectively at day 10 post infection. No significant inhibition was observed against clades A, C, D, and E. Pre-immune mouse sera had no effect on infectivity and the positive control mAb P1 inhibited infectivity of clades A, B, C, D, and E by 98, 34, 98, 100, and 98 %, respectively.

Table V. Percent inhibition of p24 levels in cultures of human PBMC infected with HIV-1 primary isolates in the presence or absence of vaccine sera. [a]

	HIV-1 Clade				
Inhibitors[b]	A	B	C	D	E
Pre-immune	0[c]	0	0	0	0
P1[d]	98	34	98	100	98
Soluble gp120	0	0	0	0	0
gp120 alum	0	33	0	4	0
gp120 conjugate	0	15	0	0	0

[a] At day 10 post-infection samples were taken and assayed in triplicate by antigen capture ELISA.
[b] Inhibitors were a 1:20 dilution of mouse sera from pools of 5 mice immunized 8 times with the particular HIV-1_{JR-FL} gp120 vaccine.
[c] Values represent the percent inhibition of control uninhibited infections.
[d] The positive control was the murine mAb P1, an anti-CD4 specific reagent.

The data presented in Tables IV and V clearly showed that the conjugation of gp120 to KHL resulted in diminished immunogenicity for the glycoprotein.

Immunogenicity Studies In Baboons:

The poor immunogenicity of the KLH-HIV-1gp120 conjugates in mice did not encourage the pursuit of a similar vaccine approach in baboons. Based in personal experience (Giavedoni et al, 1993) and literature search (Jiao et al, 1992; Letvin et al, 1997; Lu et al, 1996), the alternative that was considered the most promising was the combination of DNA immunization followed by subunit booster vaccination with the purified gp120 preparations.

Eight baboons were divided into two groups of four animals each. Group 1 baboons (animal numbers 8288, 8937, 9069, and 9167) received a mixture of 25 DNA plasmids encoding HIV-1_{LAI} env, gag, and pol genes. Group 2 baboons (animal numbers 8212, 8292, 8962, and 9186) received the same 25 DNA plasmids and two plasmids containing the genes

8962, and 9186) received the same 25 DNA plasmids and two plasmids containing the genes for IL-12 and GM-CSF. It has been reported that the route of DNA administration is in part responsible for differences in the type of immune response elicited (humoral versus cellular) (Feltquate et al, 1997). In order to minimize this variability factor, the same DNA mixture was administered by the intramuscular (IM), intradermal (ID), and "gene gun" routes according to the following schedule:

Injection site	Route	Dose/Volume
Thigh, left and right	IM	500 μg DNA / 0.240 ml divided into 2 sites
Abdominal skin	ID	200 μg DNA / 1.0 ml divided into 5-6 sites
Ear lobe	Gene gun	15 μg DNA / 0.1 ml divided into 6 sites

Animals were immunized with DNA at weeks 0, 14 and 25. On week 40, two additional baboons were included (animal numbers 8046 and 9347) as DNA-naïve controls. At that time point, all 10 animals received an IM injection containing a mixture of 50 μg of purified HIV-1_{LAI} gp120 and 50 μg of purified HIV-1_{JR-FL} gp120 in alum solution. Blood samples were taken every 4 weeks, and lymph nodes biopsies were performed at the time of the subunit immunization and two weeks later.

Studies of humoral immunity:

Plasma samples from baboons were analyzed for the presence of HIV-1gp120-specific antibodies by indirect ELISA. Disposable sterile 96-well flat bottom polystyrene ELISA plates were coated with 100 ng of HIV-1_{LAI} or HIV-1_{JRFL} gp120 in carbonate/bicarbonate buffer pH 8.5. Unabsorbed sites were blocked by the addition of 5% non-fat milk in phosphate buffer saline (M-PBS). Baboon plasma was diluted 1:400 in M-PBS. Specifically bound antibodies were detected by the addition of a 1:4,000 dilution of a goat anti-monkey IgG or IgM, labeled with horseradish peroxidase (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD). The assay was developed by the addition of TMB, and the reaction was stopped by the addition of SO_4H_2. The optical density was measured at 450nm (Shearer et al, 1998).

The ELISA assay failed to detect the generation of HIV-1gp120-specific antibodies after all three DNA immunizations. Only after vaccinating baboons with the soluble mixture of HIV-1_{LAI} and $_{JRFL}$ gp120 in alum antibody titers started to rise (Figure 4). However, naïve baboons had a similar kinetics of antibody production, indicating that there was no evidence of an anamnestic antibody response in animals that received the DNA vaccination. For all animals, antibody titers started to decline by week 8 after the subunit vaccination. Similarly to studies in

mice, antibodies to HIV-1_{JRFL} gp120 appeared earlier and reached higher titers than antibodies to HIV-1_{LAI} gp120.

Studies of cellular immunity:

The generation of memory CD4+ T cells was studied in immunized animals by analyzing proliferation of T cells upon antigen stimulation. We developed a non-radioactive technique based on flow cytometry. Baboon peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque centrifugation and resuspended at 10^6 cells/ml. An aliquot of 200 μl of cells was added to each of 9 wells in a row of a round bottom 96-well plate. Purified recombinant HIV-1_{LAI} gp120 was added at a final concentration of 1μg/ml to the first 3 wells (Antigen stimulation). Concanavalin A was added at final concentration of 5 μg/ml to the second 3 wells (Mitogen stimulation). The last three wells were not treated (Control). The plate was incubated at 37°C for 5 days. On day 5, cells were stained with the following antibody mixtures:

- Antibody Control: a mixture of normal mouse Igs labeled with FITC, PE and PECy5.
- T cells: anti-CD3 FITC (Biosource), anti-CD4 PE (Becton-Dickinson), anti-CD8 PE-Cy5 (Caltag)
- Stimulation: anti-CD3 FITC (Bios.), anti-CD25 PE (Caltag), anti-CD8 PE-Cy5 (Caltag)

CD25, also known as the α chain of the IL-2 receptor, is upregulated upon activation of T cells. An example of this analysis can be seen in Figure 5, in which PBMC from animal 8212 were analyzed 4 weeks after vaccination with the subunit preparation. Cells were run in a Becton Dickinson FACScan flow cytometer, with a first gate for lymphocytes based on forward versus side light scatter plot. A second gate was set for CD3+ lymphocytes on a forward versus FITC plot. The upper row contains the plots corresponding to the T cell subsets, where the second one (Antigen) shows the reduction in CD4 staining due to the competitive binding of HIV-1 gp120. The lower row depicts the proliferation plots, in which the third one (Mitogen) clearly shows the upregulation of CD25 in both CD4+ and CD8+ T cells by Con A. However, there is no difference in the level of activation of T cells when the Control and Antigen plots are compared. A similar outcome was found for all animals after every DNA immunization and even after 8 weeks post-subunit vaccination, indicating lack of generation of memory CD4+ T cells.

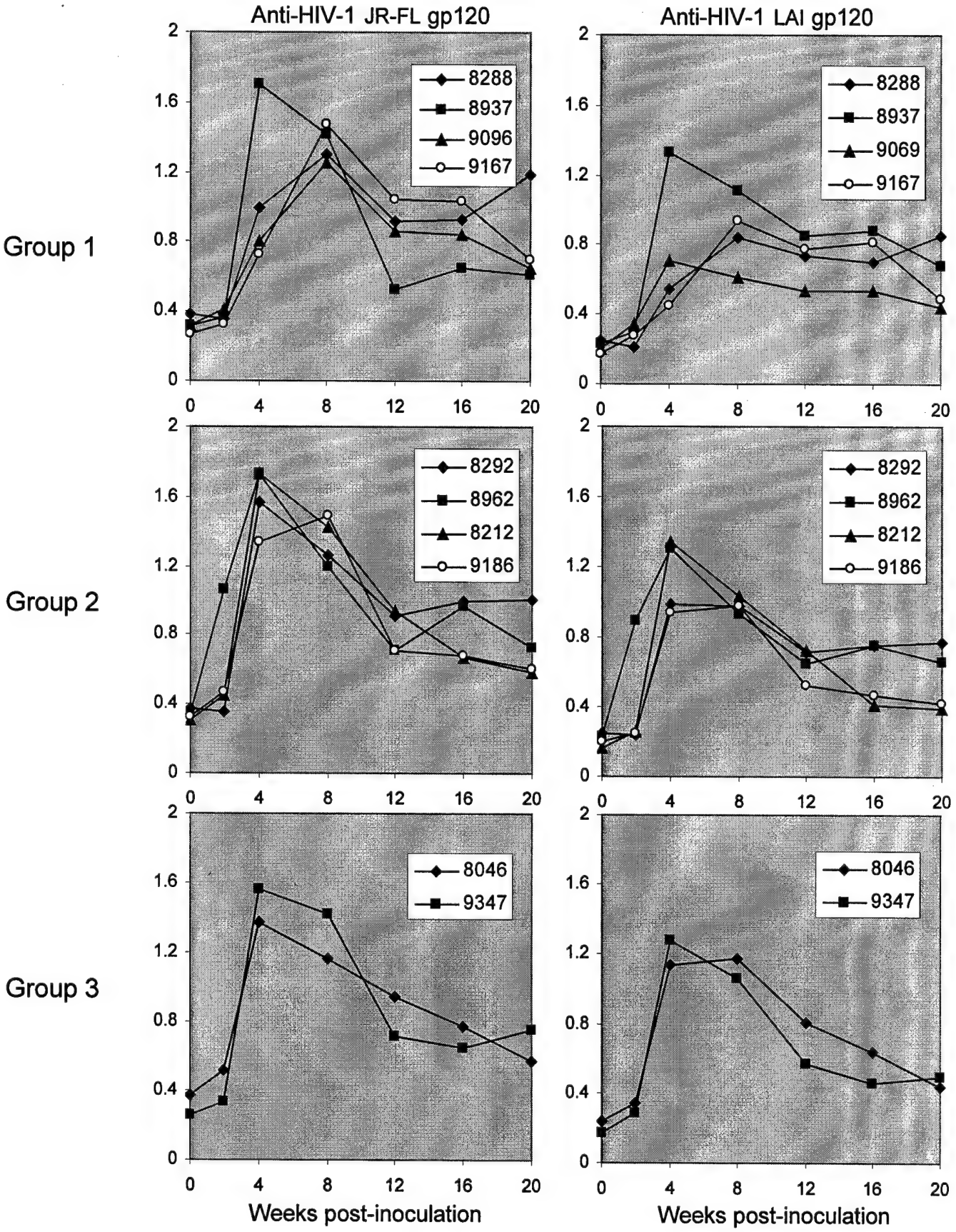

Figure 4. Anti-HIV-1 $_{JRFL}$ gp120 and Anti-HIV-1$_{LAI}$ gp120 IgG responses after booster immunization of baboons with a mixture of HIV-1$_{LAI}$ and HIV-1$_{JRFL}$ gp120 preparation. Group 1: HIV DNA vaccine. Group 2: HIV and IL12/GM-CSF vaccine. Group 3: DNA-naïve control.

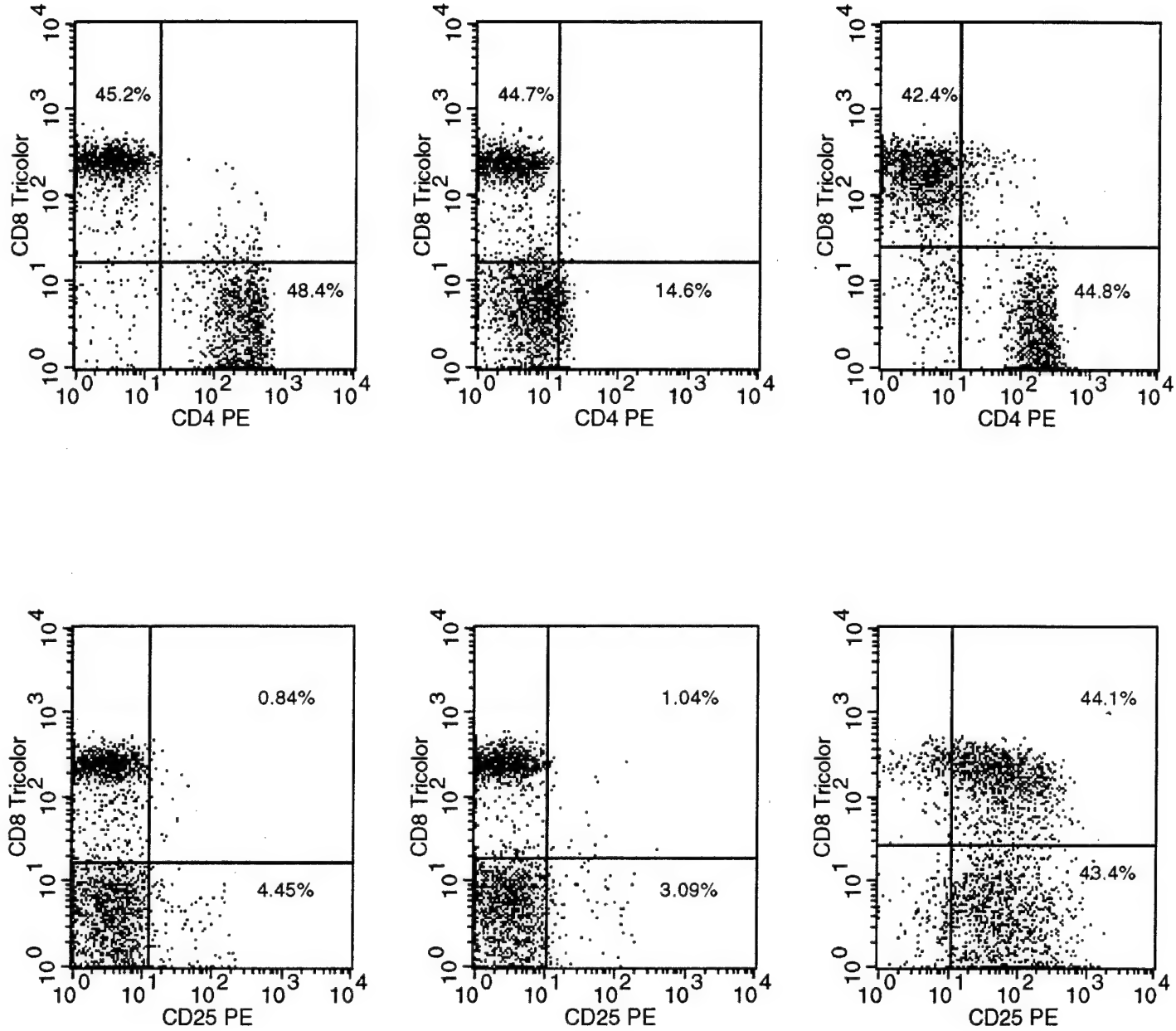

Figure 5. Flow cytometric analysis of CD3+ lymphocytes from baboon 8212 (group 2) eight weeks after HIV-1 gp120 vaccination. Cells were analyzed after five days in culture (control), or after stimulation with HIV-1_{LAI} gp120 (antigen) or Concanavalin A (mitogen).

Histopathological analysis of lymph nodes:

Lymph nodes (LNs) are the organs where antigen recognition and antibody production occurs (Garside et al, 1998). LNs were obtained from the eight baboons immunized with DNA, and the two naïve controls, before and 2 weeks after subunit vaccination. The LN taken at 2 weeks post-HIV-1gp120 vaccination were obtained from the draining area where the alum vaccine was applied. LNs were fixed in paraformaldehyde, embedded in paraffin, and sections were stained with hematoxiline-eosine. Generally, there was little change in the histology of the LNs after vaccination. However, more LNs were reactive and reticular endothelial (RE) hyperplasia was more evident. The most significant changes were observed in baboon 8962, with moderate RE hyperplasia, minimal subcapsular infiltration and generalized lymphadenopathy (data not shown). Interestingly, this animal was the one that showed the highest antibody titers at 2 weeks post-vaccination (Figure 4).

DISCUSSION

We successfully expressed high levels of the recombinant envelope glycoprotein gp120 of HIV-1_{LAI} and HIV-1_{JR-FL} strains, and developed a highly effective, nondenaturing purification method. The expressed gp120 recombinant proteins were highly pure as demonstrated by SDS-PAGE analysis. They were shown to retain their ability to bind to soluble CD4 in an ELISA format, and to cell surface-bound CD4 as determined by FACS analysis. Immunizations of guinea pigs with unconjugated gp120 derived from HIV-1_{LAI} and HIV-1_{JR-FL} in alum or in Ribi Detox adjuvants yielded relatively high serum anti-gp120 antibody titers which increased following sequential immunizations reaching a maximum of 1:250,000 to 1:500,000. Importantly, the gp120-induced immune responses also inhibited the binding of gp120 to CD4.

Conjugates of these HIV-1 gp120 preparations were prepared with tetanus toxoid (TT) with the hypothesis that very immunogenic carrier proteins such as TT will increase the immunogenicity of gp120. Crosslinking procedures were selected that did not affect the ability of HIV-1 gp120 to bind to CD4. Interestingly, the purified gp120 conjugated to tetanus toxoid elicited only a moderate anti-gp120 antibody response in BALB/C and C57BL mice. At this point, concerns originated with the issue of immunosuppression induced by the carrier protein in these conjugates. Some studies performed in inbred strains of mice indicated that priming of the immune response to a specific carrier protein suppressed the ability of the animal to

respond to the epitope associated with the haptenic portion following a subsequent immunization with a hapten-carrier protein (Herzenberg et al., 1980; Schutze et al., 1985). However, other studies indicated that priming with the carrier protein either enhanced or suppressed the immune response to the polysaccharide (Ps) moiety following a subsequent vaccination with Ps-carrier protein conjugate vaccines (Schneerson et al., 1984 Vella and Ellis, 1991 Granoff et al., 1993; Peeters et al., 199 1; Lieberman et al., 1993). Overcoming carrier protein-induced immunosuppression has been observed by utilizing alternative carrier proteins, and by modifying the dose administered in subsequent vaccinations (Gaur et al., 1990). In addition, the age of the individual at the time of immunization also represented a parameter associated with carrier protein priming and suppression (Granoff et al., 1993, Lieberman et al., 1993). Interestingly, a number of investigators have noted that carrier suppression can be bypassed in humans by modifying the carrier protein (Marcinkiewicx et al., 1992) or by performing appropriate dosing studies (Di John et al., 1989). Vaccinations with higher doses have been shown to successfully eliminate carrier suppression and significantly boost and stabilize the desired anti-pathogen responses (Di John et al., 1989). In addition, whether the antibodies to the carrier protein were actively induced or passively acquired from the mother during pregnancy has also been reported to affect the ability of infants to respond to subsequent conjugate vaccination (Barrington et al., 1994). Studies have suggested that high titers of maternally-derived passive antibodies against *Salmonella* flagellin inhibited the ability of the neonates to respond to a *Salmonella* vaccine, while neonates lacking antibodies responded as well as adults. Specific immunosuppressive effects of transplacentally acquired antibodies to diphtheria toxin and poliomyelitis have also been reported in infants. Whether this suppression with glycoconjugate vaccines results from clonal competition by carrier-reactive B-lymphocytes (Schutze et al., 1989) or by the induction of suppressor T-lymphocyte activities (Herzenberg et al., 1980) remains to be determined. Thus, the question related to the effects of pre-existing antibodies to the carrier moiety on the ability to respond to a subsequent vaccination with a conjugate formulation remains unclear and is problematic for developing an HIV-1 gp120 conjugate vaccine.

Because of this low immunogenicity, and the possibility of immunosuppression mediated by pre-existing antibodies to tetanus toxoid in humans, the purified gp120 was conjugated to KLH. SDBP- and glutaraldehyde-crosslinked gp120-KLH conjugates demonstrated good CD4-binding activity in a conjugate-specific ELISA. The extent of gp120 crosslinking in these conjugates was determined to be 50%-90% by SDS-PAGE. However, immunization studies in mice showed that anti-gp120 antibody levels were lower in animals immunized with conjugates

compared with animals receiving HIV-1 gp120 in alum alone. This is an indication that the conjugation process affects the folding of gp120 in a way that is not detected by CD4 binding studies. Another issue is the lack of crossreactivity, or clade-specificity, of the neutralizing antibodies elicited by the HIV-1 gp120 preparations. Neutralizing antibodies elicited by the clade B HIV-1_{JRFL} gp120 in alum were only able to neutralize partially HIV-1BZ167, another clade B HIV-1 isolate (33% neutralization, Table V). However, an anti-CD4 murine monoclonal antibody had only a 34% level of neutralization for that particular isolate. This may indicate that HIV-1 BZ167 is a peculiar virus that binds to CD4 in a different way as compared to other HIV-1 clade B isolates.

The lack of immunogenicity of the HIV-1gp120 preparations was a concern when planning antigenicity tests in baboons. In general, subunit vaccines, like killed vaccines, do not provide endogenously synthesized proteins and, therefore, do not induce cytotoxic T cells. Some exceptions to this limitation in CTL priming have been found; for example, subunit antigens that aggregate into virus-like particles and glycoproteins that are incorporated into lipid micelles have been demonstrated to induce antigen-specific CTLs (Tobin et al, 1997). In contrary to inactivated and subunit vaccines, live-attenuated vaccines provide for long-lasting humoral and cell-mediated immunity. However, in some cases these vaccines may be immunosuppressive, cause clinical disease if not attenuated sufficiently, or be ineffective if attenuated too much. First described in 1990 (Wolff et al, 1990), the direct injection of genes encoding protective antigens into the host has the potential to solve many of the shortcomings of the new generation vaccines. Genetic vaccines, or the use of antigen-encoding plasmid DNA to vaccinate, represent a new approach to the development of subunit vaccines (Wang et al, 1993). Additionally, the coexpression of certain costimulatory cytokines can augment the immune response to plasmid DNA immunogens. For example, coexpression of GM-CSF and/or IL-12 restored CTLs response to a nonimmunogenic influenza virus nucleoprotein (NP) mutant (Iwasaki et al, 1997); co-inoculation in mice of plasmid DNAs encoding HIV-1 envelope and IL-12 resulted in enhanced HIV-1-specific cell-mediated immunity (Tsuji et al, 1997). Furthermore, we have shown before that a combination of primary immunization with a live vector and booster inoculations with subunit vaccine induces higher antibody titers than several inoculations with live vector or subunit vaccine alone (Giavedoni et al, 1993). Therefore, we designed a vaccination schedule for baboons that included DNA immunization followed by subunit boosting vaccination.

The DNA vaccine was an HIV-1 expression library (Johnston and Barry, 1997), consisting of a mixture of 25 plasmids coding for different fragments of HIV-1_{LAI} gag, pol and env. With the idea of testing for the immune-enhancing activity of some cytokines, half of the baboons were co-

inoculated with plasmids coding for IL-12 and GM-CSF. Finally, in order to minimize the influence of the route of DNA administration, the animals were immunized by intradermal, intramuscular (Th1-inducing) and gene gun (Th2-inducing) inoculation. Unexpectedly, studies of humoral and cell-mediated immunity failed to show the generation of an immune response to HIV-1 antigens. Anti-HIV-1 gp120 antibodies were detected only after vaccination with purified HIV-1gp120 in alum, and titers started to drop after two months post-vaccination. Differences in antibody titers for the three groups of animals were not statistically significant, indicating that the DNA immunization and/or the cytokines were not effective in priming the immune system for recognizing HIV-1 gp120. Lymph node biopsies also failed to show consistently that the organs of the DNA-immunized baboons reacted more promptly to the subunit vaccination than DNA-naïve animals. Similarly to the studies in mice, the HIV-1_{JR-FL} gp120 preparation was the most immunogenic one in baboons.

KEY RESEARCH ACCOMPLISHMENTS

- Large quantities of highly pure, recombinant HIV-1_{LAI} gp120 and HIV-1_{JR-FL} gp120 were obtained.
- HIV-1 gp120 were shown to bind properly to CD4.
- Recombinant HIV-1 gp120 preparations were immunogenic in mice.
- Conjugation of HIV-1 gp120 to carrier proteins tetanus toxoid and keyhole limpet hemocyanin reduced the immunogenicity of HIV-1 gp120.
- Baboons primed with DNA vaccines coding for HIV-1 antigens followed by booster immunizations with recombinant HIV-1 gp120 failed to generated sustained antibody titers or cell-mediated immune responses to HIV-1.

REPORTABLE OUTCOMES

Manuscripts:

- Shearer, M. H., D. K. Timanus, P. A. Benton, D. R. Lee, and R. C. Kennedy (1998). Cross-clade inhibition of human immunodeficiency virus type 1 primary isolates by monoclonal anti-CD4. *J Infect Dis* 177:1727-1729.

Development of cell lines, tissue or serum repositories:

- Sera from mice immunized with recombinant HIV-1 gp120 (Dr. R. Kennedy, University of Oklahoma).
- B-cell lines derived from the 12 baboons included in these experiments (Dr. Giavedoni, SFBR).
- Plasma samples derived from baboons (Dr. Giavedoni, SFBR).

CONCLUSIONS

Despite our effort and success in producing large amounts of highly pure gp120 preparations, immunogenicity studies with these proteins have been disappointing. The main reason is that the HIV-1 envelope glycoproteins have evolved to be inefficient at eliciting protective antibody responses (Wyatt and Sodroski, 1998). A closer look at the biology of the virus and at the natural infection can produce some clues. In the infected cell, the envelope glycoprotein gp160 is synthesized, heavily glycosylated, and assembled into trimeric complexes (Chan et al, 1997). The gp160 trimers are then cleaved in the Golgi apparatus by cellular proteases into the exterior envelope glycoprotein gp120 and the transmembrane glycoprotein gp41. The gp120 and gp41 glycoproteins are maintained in the assembled trimer by relatively labile noncovalent interactions between the gp41 ectodomain and discontinuous structures composed of NH_2- and COOH-terminal gp120 sequences (Helseth et al, 1991). During natural infection, disassembled envelope glycoproteins elicit antibodies that bind to regions of gp120 and gp41 that are not exposed in intact viral particles, therefore lacking neutralizing activity.

Infection of cells by HIV-1 involves the interaction between the viral gp120 glycoprotein and the cellular CD4 glycoprotein and members of the chemokine receptor family. The gp120 binding sites for these co-receptors are not easily available to the immune system. CD4 binding occurs with elements recessed in the gp120 core, which are flanked by variable regions that exhibit extensive glycosylation (Wyatt et al, 1999). This CD4-gp120 binding then results in conformational changes that allow for the subsequent binding of gp120 to the chemokine receptors or gp41, or both (Wyatt and Sodroski, 1998). This means that epitopes likely to induce neutralizing antibodies are not normally present in the intact virion, but they form transiently during the process of virus-cell fusion. This fact has been recently probed (LaCasse et al, 1999). Researchers created two cell lines, one expressing the HIV-1 gp160 glycoprotein and the second one expressing human CD4 and the chemokine receptor CCR5. Cells were

mixed, then fixed with formaldehyde while fusing, and then used as immunogen in mice. Mice generated antibodies that were able to neutralize HIV-1 strains from five different clades.

In summary, purified recombinant gp120, although authentic in aminoacid sequence and glycosylation, does not resemble the gp120 structure that is present in the intact viral particle or in the virus-cell fusion complex. An effective vaccine against HIV-1 should be able to induce high titers of neutralizing antibodies and cytotoxic T lymphocytes. In our opinion, vaccines based solely on purified recombinant gp120 will not provide for either one.

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